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Targeting receptor-mediated endocytotic pathways with nanoparticles: rationale and advances

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Abstract

Targeting of drugs and their carrier systems by using receptor-mediated endocytotic pathways was in its nascent stages 25 years ago. In the intervening years, an explosion of knowledge focused on design and synthesis of nanoparticulate delivery systems as well as elucidation of the cellular complexity of what was previously termed receptor-mediated endocytosis has now created a situation when it has become possible to design and test the feasibility of delivery of highly specific nanoparticle drug carriers to specific cells and tissue. This review outlines the mechanisms governing the major modes of receptor-mediated endocytosis used in drug delivery and highlights recent approaches using these as targets for *in vivo* drug delivery of nanoparticles. The review also discusses some of the inherent complexity associated with the simple shift from a ligand-drug conjugate versus a ligand-nanoparticle conjugate, in terms of ligand valency and its relationship to the mode of receptor-mediated internalization.

Keywords

Endocytosis; Drug Delivery; Cellular Targeting; Multivalent Targeting

1. Introduction

The concept of the use of receptor targeting for selective drug binding and internalization has been attractive since early advances in cell biology began to reveal built-in internalization pathways that enabled selective uptake of essential constituents from the external environment. The exploitation of these endogenous uptake pathways for drug delivery began to be tested in the 1980s and 1990s, in particular with findings on the properties of tumor cells that enabled identification of receptors that were overexpressed in rapidly proliferating cells. Some of the earliest approaches utilized the transferrin receptor (TfR), a receptor binding the glycoprotein, transferrin (Tf), which sequesters free iron in the serum, and facilitates its entry into cells. TfR is selectively enriched in proliferating tumor cells [1], an observation which was subsequently used to direct selective internalization of a host of transferrin-toxin and transferrin-DNA conjugates, directly or encapsulated in liposomes or other structures, in a large body of work summarized in the excellent review by Widera *et al.* [2]. TfR remains a major drug delivery target, both in tumors as well as for

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enabling transcytosis of conjugates across barrier epithelial and endothelial cells. Another early target, the low density lipoprotein receptor (LDLR) has been of continuous interest since the early demonstration of LDL-mediated targeting of liposomes to leukemic lymphocytes in 1985 [3]. However, the repertoire of other prospective receptor targets for internalization of conjugates has significantly increased since this early work.

Interest in receptor targeting for selective uptake and internalization of drugs has expanded even further with the advent of new macromolecular drugs including DNA, peptides and proteins, because of the limitations in their ability to access vesicular or cytosolic targets. The availability of sophisticated nanotechnology approaches to encapsulate drugs, providing controlled release capacity as well as protection of macromolecules from degradation prior to reaching the site of action, has provided an additional level of complexity, since the physical properties of the particle as well as the surface composition are altered with the addition of targeting moieties to influence uptake, sometimes in unpredictable ways.

Our objective in this review article is to highlight recent advances in drug delivery that combine receptor-mediated endocytotic targeting strategies with nanomaterials, with a focus on applications that have shown promise of efficacy beyond simple cell culture into organismal models and which are poised for future translation for use in human medicine. Indeed, the use of a nanomaterial scaffold prompts concerns regarding ligand spacing and valency, which are discussed from the perspective of how they affect the internalization process. In this rapidly evolving field, other recent and excellent reviews have also provided comprehensive analyses of the importance of diverse cell biological endocytotic pathways to drug uptake and internalization [4] and on the interaction of diverse nanomaterials with cells and their preference for internalization through different endocytotic pathways [5], so we do not focus on these areas.

2. Cellular internalization pathways

It is widely accepted that endocytosis is the predominant route of uptake of macromolecules, whether they are soluble cargo or membrane proteins, into cells. In drug delivery applications, the endocytotic pathway has always held significant promise for the targeted delivery and uptake of therapeutic macromolecules into cells. However, in the past 25 years, progress in exploiting the endocytotic pathway for drug delivery has come in fits and starts, likely due to the complexity of this process as well as the complexity of the diseases that are targeted by this approach, and how these diseases may in turn alter endocytotic trafficking. This first section will review the fundamentals of endocytosis and post-endocytotic trafficking.

In 1987, the extent to which the function and mechanism of endocytosis was understood is illustrated in a review by Pearse and Crowther [6]:

“If all kinds of molecules were to enter coated vesicles on a random basis, the components of the different compartments would rapidly intermix. However, on the plasma membrane at least, selected membrane components are concentrated in coated pits, while other molecules characteristic of the parent membrane are excluded. Thus, the formation of this structure controls which proteins are transferred to another compartment and which remain behind.”

Moreover, the critical major protein components of the currently best-understood endocytotic pathway, the clathrin-mediated endocytotic pathway, were thus rather amorphously described:

“We use the term “100-kd-50-kd proteins” to denote all the accessory polypeptides. A “cage” is a polyhedral structure of complete or incomplete clathrin triskelions,

whereas a “coat” is a clathrin assembly that also contains 100-kd-50-kd accessory proteins.”

Even the pioneering work of Brown and Goldstein on the LDLR, the best-characterized endocytosing receptor at the time, was still at a nascent stage [7]. Progress in the investigation of endocytosis over the past 25 years has resulted in the characterization of a number of distinct endocytotic pathways, some of which are illustrated in Figure 1. However, the commonalities observed among the different types of endocytotic pathways have established the following paradigm for endocytosis [8–10]. The formation of an endocytotic membrane vesicle or tubule is mediated by cytosolic coat proteins, which are nucleated and polymerized at specific sites on the plasma membrane to drive subsequently the deformation of the plasma membrane, ultimately to form an endocytotic vesicle. Simultaneously, coat proteins serve as “selection devices” that determine what cargoes are to incorporate into a nascent endocytotic vesicle, such as a receptor bound to its ligand. The culmination of these processes results in the cargo being sequestered within a coated membrane vesicle or tubule.

On the other hand, despite the paradigmatic commonalities, multiple distinct endocytotic pathways have been characterized over the past 25 years on the basis of a combination of the following criteria:

1. Distinct endocytotic pathways are ultrastructurally distinct; that is, different endocytotic vesicles “look” distinct when visualized by electron microscopy.
2. Distinct endocytotic pathways are pharmacologically distinct; they are inhibited by different classes of drugs.
3. Biochemical studies show that coat protein composition is distinct for the different endocytotic pathways, and, as a corollary, gene mining has revealed novel genes encoding distinct coat proteins or genes encoding coat proteins that are related to those that are already known, thus representing potential subsets of previously characterized endocytotic pathways.
4. The cargo (membrane protein, or receptor plus ligand) incorporated into nascent endocytotic vesicles of distinct endocytotic pathways is distinct, and the post-endocytotic trafficking of the endocytosed cargo may differ, depending upon the combination of cargo and coat proteins involved in the recognition of specific cargoes. These cargoes can then be used as markers for their respective endocytotic and post-endocytotic pathways. However, it is not unusual for a specific cargo to be found within multiple endocytotic pathways, as mentioned below. In addition, small ras-like GTPases, the families of ras-like proteins from brain (Rabs) and ADP-ribosylation factor (Arf) proteins are involved in the biogenesis of organelles and the regulation of post-endocytotic trafficking of receptors [11], and are also used as markers of specific post-endocytotic organelles, as mentioned below.

For a summary of all endocytotic pathways characterized to date, the reader is referred to the following excellent reviews [8–13]. This review will henceforth focus on those receptors, endocytotic pathways, and post-endocytotic pathways that have been most effectively harnessed for drug targeting and uptake. An overview of these pathways is shown in Figure 1.

2.1. Clathrin-mediated endocytosis and post-endocytotic trafficking

Clathrin-mediated endocytosis, historically referred to as “receptor-mediated endocytosis,” is the best characterized endocytotic pathway, and is the pathway upon which the paradigm for the mechanistic basis of all endocytotic pathways is based [9]. Fundamental components

of the clathrin coat protein complex, known as adaptor proteins, are recruited to the cytosolic face of the plasma membrane where they bind to the cargo membrane protein to be endocytosed. The recruitment appears to be regulated by local changes in lipid components. The cargo to be endocytosed encompasses a large spectrum of plasma membrane proteins, such as nutrient receptors, membrane transporters, cell adhesion proteins, and signaling receptors. The clathrin-mediated pathway is also co-opted by bacteria and viruses to enter their target cells, suggesting that this pathway can internalize a wide variety of cargoes and can also accommodate significant variety in the sizes and shapes of endocytosed cargo [9, 14]. The adaptors also engage clathrin, the major coat protein that ultrastructurally defines this endocytotic pathway. Both clathrin and adaptors recruit accessory proteins to the site. This process is iterative, resulting in the localized polymerization of a coat complex that will drive the deformation of the membrane into an endocytotic vesicle and will define the shape of the nascent vesicle or tubule that encompasses and concentrates the cargo. These protein-protein interactions may also catalyze the interaction of the nascent endocytotic vesicle with the actin cytoskeleton that helps result in its fission from the plasma membrane.

Upon formation of a coated vesicle, the lumen begins to acidify, due to the primary active transport of H^+ by the vacuolar H^+ -ATPase incorporated into the coated vesicle. The coated vesicle is then uncoated by members of the heat shock protein family, thereby allowing the uncoated vesicle to fuse with the early endosome, the first major organelle along the post-endocytotic pathway. Intra-organellar acidification continues within the early endosome, dropping the luminal pH to 6.8-6.5. From the early endosome, membrane cargoes are sorted to one of a number of major post-endocytotic pathways: the recycling pathway, the degradative pathway, the retrograde pathway, or the transcytotic pathway. The early endosome is also the site at which the lowered pH causes the dissociation of some receptors from their ligands and begins the activation of hydrolytic enzymes. The small GTPases Rab5 and Rab4 regulate trafficking through the early endosome.

Cargoes sent to the degradative pathway, such as the epidermal growth factor receptor (EGFR) bound to its ligand, EGF, will be trafficked through a series of increasingly acidic organelles, the late endosome/multivesicular body (pH 6.2-5.2) and the lysosome (pH 5.2-4.5). The ever-decreasing luminal pH of these organelles will provide the environment to effect the continued dissociation of ligands from their receptors and the continued activation of luminal acid-dependent hydrolases. The major classes of lysosomal acidic hydrolases are proteases, lipases, phosphatases, sulfatases, and glycosidases. These hydrolases are involved in the degradation of endocytosed cargo, such as receptor ligands and possibly the receptors themselves. This pathway effectively represents a terminal pathway for endocytosed cargo.

Many drug delivery strategies have employed the pH change within endosomes and post-endosomal compartments like late endosomes and lysosomes as a trigger to activate conformational changes encompassed in delivery vehicles that trigger the leakage of endocytosed cargo to cytosol from these acidic compartments, by harnessing "endosomolysis". Examples of agents sensitive to such pH changes include polymers [15–17], pH sensitive nanogels [18, 19], as well as proteins critical to pathogen infection such as the hemagglutinin glycoprotein from influenza [20], listeriolysin [21], melittin [22], and many other viral proteins. These and many others have been creatively employed in a host of drug delivery strategies over the past several decades, with varying success. Likewise, the hydrolytic constituents of the lysosomes in particular have been exploited as triggers for release of active drugs from prodrugs.

Cargoes sent to the recycling pathway may transiently reside in a distinct endosomal compartment, the recycling endosome, whose luminal pH is approximately 6.5-6.4, prior to

their reappearance at the plasma membrane. Depending upon the receptor, the rate at which recycling occurs may be very rapid, such as with the transferrin receptor (TfR), or more slowly, as with certain G-protein coupled receptors (GPCR) [23]. Rab11 and arf6 are major regulators of sorting at the recycling endosome. Along the way, with the TfR as a prototypical example, the receptor's ligand will have dissociated from the receptor, typically in the early endosome, and the receptors are recycled to the plasma membrane as empty receptors.

Certain membrane proteins are trafficked along the retrograde pathway to the trans-Golgi network (TGN), ultimately to be recycled to the secretory pathway, where they are involved in secretory vesicle formation or in the biogenesis of lysosomes. Proteins following such a pathway would include proteins that contribute to the contents of secretory vesicles or lysosomes, such as the mannose-6 phosphate receptor, or proteins involved in vesicle fusion with the plasma membrane, the receptors for soluble N-ethylmaleimide sensitive factor attachment proteins (SNARE). In addition, some bacterial toxins are retrogradely sorted to the TGN, such as Shiga toxin B (StxB) and cholera toxin B (CtxB), and require further retrograde transport to the endoplasmic reticulum (ER), where they are then exported into the cytosol to exert their toxicity on cytosolic targets [24, 25]. For some endocytosed cargo, the final destination along the retrograde pathway will even be the nucleus, although it is not clear that this final destination occurs under normal conditions. In cancer cells, the EGFR together with the transmembrane mucin, MUC1, have been shown to be retrogradely trafficked to the ER, exported to the cytosol, and imported into the nucleus for a role in transactivation [26].

Polarized cells, in particular epithelial and endothelial cells, possess another post-endocytotic trafficking pathway, the transcytotic pathway. Macromolecular ligands or cell surface proteins are ferried intact across the cell, in an apical-to-basolateral or basolateral-to-apical (for endothelial cells, luminal-to-abluminal) direction, via multiple vesicular intermediates that traverse multiple endosomal compartments. Select transmembrane receptors internalized via clathrin-dependent endocytosis and lipid rafts have been identified as cargo for the transcytotic pathway [27–30]. There have been many excellent reviews on the cell biology of transcytosis, so it will not be covered in significant detail here [2, 28, 31–38].

However, it is instructive to describe briefly the best-characterized basolateral-to-apical transcytosing receptor, the polymeric immunoglobulin receptor (pIgR) and the best-characterized apical-to-basolateral transcytosing receptor, the neonatal Fc receptor (FcRn), both of which initiate their transcytotic journeys through clathrin-dependent endocytosis. Studies centered around these receptors as markers of transcytotic pathways have resulted in the mechanistic characterization of the regulation of transcytosis across epithelia and endothelia [28, 32]. The pIgR mediates the basolateral-to-apical transcytosis of polymeric immunoglobulins, such as dimeric IgA or pentameric IgM, in the defense of mucosal surfaces.

The pIgR is delivered to the basolateral domain from the TGN. At the basolateral membrane, it may bind to its ligand, and irrespective of ligand occupancy, it is endocytosed in a clathrin-dependent fashion. The receptor is then sorted through the basolateral early endosome, the common recycling endosome, to the apical recycling endosome, and finally trafficked to the apical membrane. Once at the apical membrane, the extracellular ligand-binding domain of the pIgR is cleaved, thereby releasing the polymeric immunoglobulin, complexed with the cleaved receptor fragment, into the external secretions. If the pIgR did not bind to ligand at the basolateral membrane, the empty receptor is still cleaved, and the

receptor fragment is released into the external secretions. The pIgR can also recycle at the basolateral and apical membranes [28].

In contrast, the FcRn can mediate bidirectional transcytosis of its ligand, immunoglobulin G (IgG) [32] (Figure 2). The functional receptor is a heterodimer comprised of FcRn and beta2-microglobulin. The basis for transcytotic transport is the pH-sensitive binding of FcRn to IgG, with a high affinity interaction occurring at pH < 6.5, but not at the physiological pH of 7.4. Thus, FcRn at the apical membrane of an epithelial cell can bind to IgG present in acidic luminal fluids, be transcytosed to the blood side, and release its cargo. This process occurs in enterocytes in the neonatal absorption of IgG from mother's milk in rodents [39, 40]. The alternate route is for the IgG to be endocytosed by fluid-phase uptake, then transported to acidic endosomes, in which the FcRn resides, and to bind to FcRn. The FcRn-IgG complex can then be transcytosed to the side contralateral to that which endocytosed the IgG. This process occurs in humans, in the fetal absorption of maternal serum IgG by transcytosis across the syncytiotrophoblast cells of the placenta [41, 42]. Some of the organelles through which the FcRn is sorted in its apical-to-basolateral transcytosis appear to be the same as those traversed by the pIgR in its basolateral-to-apical transcytosis [43], while there also appears to be differential regulation of each receptor through their respective pathways, and even differential regulation of the trafficking of the FcRn through either of its polarized transcytotic pathways [44].

However, the designation of FcRn as a “neonatal” receptor is a misnomer, as there is significant expression of the FcRn in adult tissues with demonstrable physiological significance in the adult animal. The FcRn is expressed in the adult in vascular endothelium, and is highly expressed in endothelial cells of the central nervous system and the choroid plexus. It is also expressed in the adult gut, kidneys, airways, and professional antigen-presenting cells, such as monocytes, macrophages, and dendritic cells. The proposed functions of FcRn at these sites are intriguing. For example, in the gut and in human intestinal epithelial cell lines, it has been shown that enterocytes can transcytose IgG in the blood-to-lumen direction, thus providing a pathway for secretion of IgG into mucosal fluids [45, 46]. FcRn-dependent immune protection against mucosal infections by IgG transcytosed into uterine/vaginal fluids [47] and gastric juice [48] has also been demonstrated. Moreover, in the gut, the apical-to-basolateral transcytosis of IgG-antigen complexes could then deliver these complexes to mucosal dendritic cells [46]. A vigorous FcRn-dependent, lumen-to-blood, transcytotic pathway exists in airway epithelium [49, 50], as well as in the brain-to-blood direction [51, 52].

Surprisingly, in addition to the IgG transport function of FcRn, studies from FcRn-deficient mice have shown that FcRn appears to prolong the half-life of IgG in the circulation and extends the half-life of serum albumin by currently uncharacterized mechanisms [53, 54].

Thus, the role of FcRn in the transport of IgG and its role in the metabolism of IgG and albumin have significant pharmaceutical relevance and impact with respect to the delivery and pharmacokinetics of Fc-fusion products and therapeutic monoclonal antibodies. Overall, the characterization of transcytotic receptors such as the pIgR and FcRn also provides for the potential development of therapies utilizing these pathways for macromolecular transport across cellular barriers.

A recent development in clathrin-dependent endocytosis has been the discovery and characterization of so-called “alternate” clathrin adaptors that work independently of, or in conjunction with, the canonical AP-2 clathrin adaptor [10]. Some of these adaptors are highlighted in the sections below on specific cell surface receptors. These alternate adaptors contribute to a higher degree of selectivity in the regulation of the endocytosis of these

receptors and highlight the potential for selective pharmacological manipulation of specific, clathrin-dependent endocytotic pathways. Indeed, a recent chemical biology approach for clathrin-dependent endocytosis has identified and enabled characterization of a drug “selective” for the inhibition of the internalization of Tf, EGF, and human immunodeficiency virus, but not of StxB [55].

To complicate matters further, cell surface receptors can be routed to different internalization pathways depending upon prevailing cellular conditions. For example, the EGFR will be internalized by clathrin-dependent endocytosis, caveolae-dependent endocytosis, or macropinocytosis, depending upon the concentration of EGF [8, 56]. A similar situation occurs with the transforming growth factor (TGF) receptor. If the TGF-receptor is internalized through clathrin-dependent endocytosis, it is recycled to the plasma membrane. However, if it is internalized through caveolae-dependent endocytosis, it is sorted into the degradative pathway [56].

It is clear that even early endosomes fed from clathrin-dependent endocytosis are distinct, relative to the eventual fates of the receptor-cargo complex. There is a dynamic, highly mobile population that matures rapidly toward late endosomes; these endosomes contain cargo such as LDL and EGF. There is also a more slowly maturing population occupied by recycling cargo, such as Tf. The fates of the receptor-ligand complexes may depend upon the adaptors that are initially recruited for the endocytosis of the specific cell surface receptor [57].

In summary, the endocytotic and post-endocytotic pathways are highly dynamic platforms to regulate the internalization and processing of internalized cargo. In addition, the pathways of internalization and processing of cargo can differ by cell types, even within the same general pathway, that will be dependent upon the differential expression of endocytotic protein complexes in each cell type. In addition, these pathways can differ according to the physiological or pathological status of the target cell, which, as illustrated below, may be used to develop targeted drug delivery strategies. In the next several sections, we illustrate the trafficking dynamics of three different receptor pathways, each of which harnesses the clathrin-mediated endocytotic pathways in a different manner.

2.1.1. Transferrin receptor (TfR) trafficking—There are two isoforms of the TfR: TfR1 and TfR2 [58], which have different trafficking mechanisms (Figure 1). TfR1 is the canonical recycling receptor that is typically described. At the plasma membrane, TfR1 binds to its ligand, Fe³⁺-loaded transferrin (holo-Tf). The receptor-ligand complex undergoes clathrin-mediated endocytosis, which is highly dependent upon its interaction with the AP-2 clathrin adaptor, and is delivered to early endosomes. In the lowered pH environment, the Fe³⁺ dissociates from holo-Tf, forming apo-Tf, and the Fe³⁺ is exported from the endosome to the cytosol by membrane transporters. However, at the pH of the early endosome, the apo-Tf remains bound to TfR1. The TfR1-apo-Tf complex is trafficked to recycling endosomes, and ultimately back to the plasma membrane. At the pH of the extracellular fluid, the apo-Tf has a low affinity for TfR1 and dissociates. The apo-Tf is available to bind to Fe³⁺ again, and the TfR is available to bind to another holo-Tf. However, ligand binding is not obligatory for internalization of TfR1.

The endocytosis of TfR1 may be modulated by accessory interacting proteins. The familial hemochromatosis protein (HFE) is a transmembrane protein and negative regulator of TfR internalization [59, 60]. HFE is highly expressed in liver and intestine. Particular mutations in HFE will result in the loss of inhibition of TfR endocytosis, resulting in cellular iron overload. Another TfR1-interacting protein, TfR trafficking protein (TTP), is a cytosolic protein that regulates TfR1 endocytosis [61]. Its depletion inhibits TfR1 endocytosis, and,

interestingly, its overexpression also inhibits TfR1 endocytosis, suggesting that the stoichiometry of TfR1 and TTP must be balanced for the optimal endocytosis of TfR1.

In the presence of holo-Tf, the endocytosis of TfR2 appears to be identical to that of TfR1. However, in the absence of holo-Tf, TfR2 is internalized and rapidly degraded via the lysosomal pathway [62]. The switch from a recycling pathway to a degradative one involves an interaction of TfR2 with a cytosolic accessory protein, phosphofurin acidic cluster sorting protein (PACS1), which does not interact with TfR1. Thus, the post-endocytotic processing of any cargo internalized by TfR may differ, depending upon the TfR isoform to which it is bound, which is in turn related to tissue expression of the different TfR subtypes and whether they are influenced by disease.

Tf (and likely the TfR itself), has been demonstrated to undergo transcytosis in gastrointestinal epithelium [63] as well as across the blood-brain barrier [64], and this transcytotic pathway has been exploited for the trans-epithelial and trans-endothelial delivery of therapeutic agents across these barriers.

2.1.2. Low density lipoprotein receptor (LDLR) trafficking—LDL receptor is a single-chain transmembrane glycoprotein responsible for binding and endocytosis of LDL, and it is the founding member of the LDLR superfamily. This receptor family includes megalin [65, 66], also known as LDLR-related protein 2 (LRP2), a multipurpose endocytotic receptor, and the vitellogenin receptor [67], involved in the endocytosis of yolk proteins. At the plasma membrane, LDLR binds to LDL, and the complex is internalized through the clathrin-dependent pathway. However, it differs from the internalization of the TfR; in addition to the AP-2 clathrin adaptor, LDLR endocytosis is dependent upon adaptor proteins from the Dab2/Numb/ARH family of proteins, which bind to the cytoplasmic domain of the LDLR (and related receptors), as well as to AP-2 clathrin adaptors and clathrin [68–70]. Once endocytosed, the LDLR is trafficked into early endosomes, where lower pH decreases the affinity of LDLR for LDL. LDL dissociates and is trafficked to late endosomes and finally to lysosomes, where the LDL is hydrolyzed into peptides, amino acids, cholesterol, and other lipid products. These are all then exported from the lysosome for utilization by the cell. The empty LDLR is recycled to the plasma membrane to mediate another round of LDL binding and internalization. The multiple ligands that bind to the LDL-related receptor, megalin, also dissociate in the lowered pH environment of endosomes, and the receptor is recycled [65].

2.1.3. Epidermal growth factor receptor (EGFR) trafficking—The EGFR is a single-chain transmembrane receptor responsible for binding EGF and activating multiple intracellular pathways, such as the well-characterized mitogenic pathway. While the endocytosis of the TfR and the LDLR is constitutive, the endocytosis of the EGFR is dependent upon ligand binding, thus linking the down-regulation of the signaling complex to ligand binding. Upon ligand binding, the activation steps of the EGFR include dimerization and autophosphorylation of tyrosine residues. In addition, the EGFR is a receptor that may be sorted through multiple endocytotic pathways, possibly depending upon the ligand concentration in the extracellular milieu. Evidence suggests that at low concentrations of ligand, the EGFR is internalized through clathrin-dependent endocytosis, and at high ligand concentrations, a clathrin-independent, raft/caveolar-dependent endocytotic pathway is also used [71]. In these cells, sorting through the clathrin-dependent pathway is primarily a recycling pathway, while sorting through the raft/caveolar pathway leads to degradation of EGFR. However, there are other observations suggesting to the contrary: the EGFR may enter both pathways irrespective of ligand concentration, and even some data that suggest that the internalization of EGFR is entirely clathrin-dependent [72]. These differences may

result from use of different cell types for analysis as well as differences in experimental approaches.

A combination of signaling and endocytotic adaptors associates at early steps with the activated EGFR. One of the proteins recruited to this early complex is c-cbl, an ubiquitin ligase that catalyzes the ubiquitination of the cytoplasmic domain of the EGFR. There is general agreement that EGFR ubiquitination regulates the endocytosis and postendocytotic trafficking of the EGFR. Ubiquitination of the EGFR at the plasma membrane allows for its interaction with and internalization by clathrin adaptors that possess ubiquitin-interacting motifs. Once at the early endosome, if EGF dissociates from the EGFR, the EGFR is de-ubiquitinated and rapidly recycled. There is evidence that ligand-bound EGFR may also rapidly recycle [73]. If the EGFR remains ubiquitinated or is re-ubiquitinated in early or late endosomes, then a distinct coat protein complex, the endosomal sorting complex required for transport (ESCRT) complex, interacts with the EGF-EGFR complex, to generate multivesicular bodies containing the EGF-EGFR complex. The multivesicular bodies ultimately fuse with lysosomes to effect the degradation of EGF and the EGFR, although the EGFR can also slowly recycle from the late endosome as well [73, 74].

Stimulation of the EGFR by high EGF concentrations also mobilizes other newly characterized endocytotic pathways, macropinocytosis or circular dorsal ruffles (described below), through which the EGFR may be internalized.

2.2. Macropinocytosis and circular dorsal ruffles

Stimulation of cells by growth factors such as EGF or platelet-derived growth factor also induces macropinocytosis and circular dorsal ruffles, two other modes of clathrin- and caveolin-independent endocytosis [75–78]. The fundamental features and effectors of macropinocytosis are being actively characterized. Macropinocytosis occurs at the periphery of adherent cells, at areas active in the formation of membrane ruffles involving dynamic actin cytoskeletal rearrangements. Occasionally these ruffles are then thought to fold back upon themselves to envelop fluid and plasma membrane domains to form endocytotic structures known as macropinosomes, with sizes ranging from 0.2 to 10 μm in diameter. Thus macropinocytosis represents a high volume endocytotic pathway for membrane and fluid internalization.

Macropinocytosis was once thought to be a somewhat artifactual phenomenon observed only in cultured cells, but growing evidence suggests that these large volume uptake mechanisms may exist in cells *in vivo* [75, 77, 78]. In addition, multiple pathogens, from protozoa to viruses, have evolved clever mechanisms to enter cells via macropinocytosis [77, 78]. Moreover, macropinocytosis has generated interest relative to drug delivery applications [79], with reports of macropinocytosis being the preferential pathway for the internalization of arginine-rich cell-penetrating peptides [80, 81], although this activity may be cell-specific [82]. Nonetheless, there is a very intriguing report on a particular arginine-rich peptide conjugated to a pro-apoptotic peptide that binds to leukemia- and lymphoma-derived cell lines and is internalized through macropinocytosis, resulting in the selective killing of these cells [83]. Given the apparent diversity of cargoes, from fluid to particles, internalized by macropinocytosis, and its capacity for high-volume uptake, this pathway will likely continue to attract attention in the drug delivery field.

Circular dorsal ruffles form on the free dorsal surface of cells, in an apparent wave-like manner [75, 84, 85]. High concentrations of EGF activate circular dorsal ruffles, with EGFR becoming concentrated in these ruffles, and EGFR then being internalized by tubular endocytotic membranes. Despite their similarities, macropinocytosis and circular dorsal ruffles appear to be distinct, as the proteins that regulate each of these pathways are different

[75]. Currently, the physiological and potential pharmaceutical relevance of circular dorsal ruffles is unknown.

2.3. Clathrin-independent endocytosis—caveolar endocytosis

Caveolae are flask-shaped, 60–80 nm-diameter invaginations of the plasma membrane and are clearly structurally and functionally distinct from clathrin-coated endocytotic structures. A major structural protein of caveolae is the integral membrane protein caveolin, of which there are 3 isoforms, caveolin-1, -2, and -3. Caveolin-1 and -2 are widely expressed, but caveolin-3 is expressed exclusively in muscle.

Caveolin adopts a hairpin structure within cellular membranes, in which its amino- and carboxy-termini are cytoplasmic, and the hairpin turn is possibly exposed extracellularly. Biochemically, caveolins oligomerize, are associated with lipid rafts, and become incorporated into so-called detergent resistant membranes, although there are also non-caveolin detergent resistant membranes. Interestingly, neurons and leukocytes are cells that do not express caveolin nor do they form caveolae. Conversely, caveolin appears to be necessary and perhaps sufficient to form caveolae. In cells that normally do not express caveolin and do not form caveolae, if caveolin-1 is ectopically expressed in these cells, caveolae will then form [86]. However, recent work suggests that another family of proteins (cavins) that associate with the cytoplasmic side of caveolae, are important in the formation of caveolae [87]. In addition, knockout organisms for cavin phenocopy knockouts for caveolin [88, 89].

While a number of signaling protein complexes have been found to associate with and are regulated by caveolae [90], there are only a few cargoes that have been demonstrated to be internalized via caveolae. Most are exogenous pathogens or fragments of pathogens, such as simian virus 40 (SV40), StxB and CtxB. These exogenous cargoes may be internalized via caveolae by virtue of their binding to specific glycosphingolipids that interact with caveolin1 and are therefore concentrated in caveolae. Endogenous cargo internalized by caveolae include sphingolipids and glycosylphosphatidylinositol (GPI)-anchored membrane proteins (GPI-AP), such as CD59, a mediator of the complement reaction [91–93], although these cargoes are not necessarily restricted to internalization by caveolae (see Section 2.4). In addition, unlike the case with clathrin-coated membranes, in cells in which caveolae have been observed in real time, only a small percentage of caveolae are dynamic and appear to be involved in endocytosis, which is also dependent upon the presence of membrane cholesterol.

The cargoes endocytosed by caveolae are delivered to either early endosomes or specific caveolin-1-containing endosomes, known as caveosomes [94]. Caveosomes are distinct from early and recycling endosomes and do not appear to contain material endocytosed by clathrin-dependent endocytosis, such as Tf. In addition, the intra-organellar pH of caveosomes is neutral, in contrast to the acidic pHs of early and late endosomes. However, recent data suggest that caveosomes are organelles that are artifactually generated by the overexpression of caveolin-1 in cells and are actually modified late endosomes [95]. Thus, caveolar cargo, such as SV40, is trafficked through typical early and late endosomes prior to being retrogradely sorted to the endoplasmic reticulum.

Endothelial cells, in particular, those lining the blood-brain barrier or lung alveoli, appear to possess numerous caveolae, which are thought to mediate transcytosis from the blood side to the abluminal side. Nitrated albumin [96] and albumin complexed to myeloperoxidase [97], and native albumin [98] have been observed to transcytose across endothelia via caveolae, likely in a receptor-mediated fashion. Perhaps one of the most convincing demonstrations of caveolae-mediated transcytosis is observed in an intravital microscopic study of the

transcytosis of antibodies against aminopeptidase P, a protein enriched in caveolae, across lung endothelial cells [99]. These results suggest that the rate of caveolae-mediated trafficking may depend upon cell type.

2.4. Clathrin independent carriers (CLIC) and GPI-anchored protein-enriched early endosomal compartment (GEEC) pathway

As its name implies, this pathway was uncovered after clathrin- and caveolin-dependent pathways were inhibited [100]. Endocytotic CLIC membranes are fairly prevalent, with a tubular or ring-like in morphology, but no ultrastructurally apparent “coat” [8]. Recent work has shown that CLICs are a high-volume internalization pathway in fibroblasts, internalizing three times the volume internalized by the clathrin-dependent pathway [101, 102]. Thus, CLICs are a major contributor to fluid and bulk membrane uptake by cells, although it is not clear that all cargoes endocytosed by CLICs traffic to GEECs. And, as is the case with caveolar – dependent endocytosis, only a few cargoes have been identified for CLIC/ GEECs. Notable among these cargoes are fluid-phase markers and GPI-APs, such as the folate receptor (FR)- α , and CtxB [101, 102], the latter of which, as mentioned above, is also a cargo for caveolae. The FR was originally thought to be a cargo for caveolae, but later was shown to enter caveolae only after cross-linking [103]. Further evidence that the CLIC/ GEEC pathway is distinct from clathrin-dependent endocytosis is that the FR sorted into GEEC does not colocalize with TfR, which is a marker of early endosomes fed from the clathrin-dependent pathway [104]. In addition, inhibition of the small GTPase Arf1 inhibits the uptake of the FR into GEEC, but does not affect the uptake of TfR. Moreover, the luminal pH of GEEC is close to 6, which is more acidic than typical early endosomes (~6.5) [105]. Finally, the delivery of the FR from GEEC into recycling endosomes is regulated by the small GTPase cdc42 [106]. Thus, traffic along the CLIC/GEEC pathway appears to be regulated by small GTPases distinct from the family of GTPases that play a predominant role in the regulation of post-endocytotic traffic in the clathrin-dependent pathway, the rab family of GTPases.

Recent additional cargoes characterized for CLIC are: major histocompatibility complex class I protein [107]; the cell adhesion molecules CD44 [102, 108] and ICAM1 [108]; the GPI-APs CD55 [108], CD59 [107], and Thy-1 (CD90) [101]; the heavy chain of amino acid transporters CD98 [101]; CD147 (emmprin) [108]; and, the glucose transporter Glut1. Thus, intriguingly, CLICs may regulate cell adhesion and cell migration [101]. However, it is not clear whether these cargoes are post-endocytotically sorted to GEECs. In addition, it is not clear whether this variety of cargoes reflects the diversity of subtypes of CLIC pathways and/or differences in post-endocytotic sorting in different cell types, particularly to GEECs. Cargo internalized via the CLIC pathway, if not sorted to the GEEC, may be alternatively sorted to recycling endosomes or lysosomes, a process that may also differ among different cell types [8].

GPI-APs are also transcytosed in a basolateral-to-apical direction in cultured MDCK cells [27] and in polarized hepatic cells [109]. The transcytotic pathway for GPI-APs appears to be distinct from cargo entering the basolateral-to-apical transcytotic pathway via clathrin-mediated endocytosis, such as for the pIgR. GPI-APs together with basolateral proteins are delivered to the basolateral membrane from the trans-Golgi network. The GPI-APs are rapidly endocytosed into caveolae-like structures, along with fluid phase markers, while basolateral proteins are not. GPI-APs are then transcytosed to the apical membrane via clathrin-free transport carriers [27].

2.5. Alterations in the internalization and trafficking of membrane receptors upon cross-linking

There are clear examples of alterations in the internalization and post-endocytotic trafficking of receptors upon cross-linking of those receptors by exogenous agents such as anti-receptor antibodies. The example of the FR was mentioned in **Section 2.4.**, and an example of EGFR trafficking being altered by the therapeutic monoclonal antibody Herceptin® is described in **Section 4.1.** On the other hand, pertuzumab, a monoclonal antibody that inhibits the dimerization of the EGFR with other HER receptors, enhances the degradative down-regulation of the EGFR [110]. Similarly, antibodies against the nicotinic acetylcholine receptor, when added, will induce the internalization of the receptor [111]. Transcytosing receptors can also be influenced by ligand valency. For the FcRn, ligand valency affects the efficiency of its transcytosis and the fraction of occupied receptors that gets diverted to lysosomes for degradation [112]. Thus, multivalency, described in **Section 5**, will likewise potentially impact the post-endocytotic trafficking of the target proteins, which may then enhance or detract from the therapeutic objective of the product.

3. Cellular targeting strategies applied to nanoparticles

The concept of efficient and targeted delivery of drug carriers to or into the target tissue or organ has historically attracted attention, especially for potent drugs with toxicity concerns. From the earliest studies exploiting selective enrichment of TfR on proliferating tumor cells to target Tf-toxin conjugates, rapid progress in cell biology, genomics and proteomics has now expanded this approach for drug delivery in a variety of circumstances; for instance, target cells can be distinguished from non-target cells by screening for specific biomarkers on the plasma membrane. Delivery of drug carriers to target cells by recognition of surface biomarkers is a promising approach with clear advantages. First, cellular specificity may reduce toxicity and keep adverse reactions to the minimum. Second, introduction of the cellular specificity may overcome the limitations of some previous therapies. For instance, implant of radioactive isotopes or passive drug delivery strategies based on the EPR effect are only applicable to solid tumors above a certain size [113]; whereas, the cellular level specificity provides the possibility of treating diseases in a variety of tumor and non-tumor tissue that are distinguished by their unique cellular properties as opposed to the tissue's physical properties.

In this section, we discuss new advances in drug delivery strategies focused on targeting nanoparticles to examples of biomarkers on the cell surface which enable their internalization, with emphasis on their in-vivo applications. These approaches are categorized by the examples of the diverse biological targets used for tissue internalization, and illustrative examples for each category are summarized in Figure 3. These are by no means comprehensive, as other excellent work has focused on use of additional receptor targets; however these targets largely have more information available on their receptor-mediated endocytotic uptake pathways as described in **Section 2**. It should likewise be mentioned that the literature has shown that the addition of targeting moieties may not influence the general biodistribution of nanoparticles in terms of their tissue deposition, but may have its greatest potential in enhancing internalization to appropriate target cells within tissues [114–116].

3.1. Nanotechnology

Nanotechnology has the potential to add remarkable value to therapeutic agents by providing the means by which drugs may be encapsulated to achieve a number of endpoints, including but not limited to controlled release, tissue targeting, penetration of barrier tissues and protection of the encapsulated drug. Additional opportunities lie in the ability of

nanotechnology to combine therapeutics spatially for co-delivery, as well as to combine therapeutic and imaging agents for “theranostic” imaging and treatment. Moreover, nanoparticle formulations offer the potential to protect encapsulated or conjugated macromolecular drugs such as siRNA or proteins from environmental degradation. A variety of nanoparticles, ranging between 1–100 nm, are commonly exploited for drug delivery. These structures include liposomes, micelles formed from polymers, protein-based nanoparticles and hybrid structures with mixed properties.

To date, the most successful clinical applications of nanoparticles for drug delivery include liposomal formulations such as AmBisome, PEG/liposome encapsulated doxorubicin such as Doxil® and other liposomal and PEGylated proteins, antibodies or small molecule drugs [117]. Use of nanoparticles largely allows these agents to achieve sustained plasma levels while encapsulated, with the liposomal size preventing the rapid clearance of drug by the kidneys that occurs with the free agent [118]. Additional benefits of the nanoparticle carrier are relevant to the apparent trapping of carriers containing drug in the interstitium of solid tumors, due to the so-called enhanced permeability and retention (EPR) effect.

The approved limited use of nanoparticles in the clinic thus far does not match the potential utility of these diverse agents, since their size is ideally suited to intracellular uptake through the endocytotic mechanisms described above. However, nanoparticle interaction with cells and the cellular internalization machinery is quite complex and it has been challenging to identify unifying principles for particle internalization via specific pathways across cell types. Nanoparticle shape, surface charge and other physical properties affect their endocytosis in ways that are not yet completely understood. This area has recently been comprehensively reviewed [5]. It has also been documented that particle charge and size significantly impact the ability of nanoparticles to traverse physiological tissue barriers such as the mucus layer [119] as well as their *in vivo* distribution [120, 121]. Encapsulation of therapeutic agents into nanoparticles with a particular affinity for an internalization pathway is potentially highly efficient mode of membrane targeting. However, most cells are not highly specialized to recognize particles alone as targets for selective uptake, with the exception of professional scavenging cells such as reticuloendothelial cells and macrophages [122, 123].

Addition of targeting ligands with specificity for receptors, either protein, carbohydrate or lipid based, to nanoparticles is an ideal strategy for targeting of nanoparticles to particular cells and/or for reaching specific membrane encapsulated compartments in cells. Membrane compartments are a desirable target for several reasons—a number of therapeutic targets for some of society’s most challenging diseases (*e.g.*, Alzheimer’s disease) are located to such compartments. Second, the gradual acidification of compartments ranging from pH 6.5 in peripheral endosomes to pH 4.5 in lysosomes offers an opportunity to activate a variety of pH sensitive triggers as discussed above; these include induction of conformational changes in pH sensitive peptides [124, 125] sufficient for membrane disruption and pH-triggered release of drugs from prodrug formulations. Finally, use of constitutive recycling mechanisms can sustain plasma levels of peptide drugs such as insulin by transiently storing and re-releasing drug, exemplified by previous work on transferrin [126]. Targeting of nanoparticles is also a strategy for accessing and reaching peripheral non-tumor tissue that may have more limited blood flow and may not be as amenable to targeting by physical methods.

3.2. Epidermal growth factor receptor (EGFR) nanoparticle targeting

Receptor tyrosine protein kinases phosphorylate tyrosine amino acid residues, and trigger downstream signal transduction. This protein superfamily includes EGFR, vascular endothelial growth factor receptor (VEGFR), insulin receptor and others. These proteins

internalize largely but not exclusively via clathrin mediated endocytotic pathway although complexities associated with concentration-dependent diversion to other pathways has been suggested as discussed in **Section 2.1.3**. The amplification, over-expression or mutation of members of the EGFR family have been demonstrated to play important roles in the pathogenesis and progression of certain aggressive types of cancer [127]. Therefore, the members of the EGFR family are ideal targets for delivering drugs to these cells. A straightforward approach for targeting EGFRs is to conjugate drug carriers with EGF. Using this approach, the use of biotinylated-EGF-modified gelatin nanoparticle carriers enhanced the aerosol delivery efficiency of cisplatin to plasma and lungs via inhalation in mice; it also significantly reduced the size of A549 cell-induced tumors [128]. However, EGF-EGFR targeting simultaneously introduces exogenous EGF with biological activity into the internal environment, and this constitutes a potential risk for clinical applications. Using antibodies to target EGFR can overcome this problem. Iron oxide magnetic nanoparticles conjugated with an antibody targeting EGFRvIII decreased the survival and EGFR phosphorylation levels in GBM cells in-vitro, but showed no toxicity in human astrocytes [129]. Their convection-enhanced delivery led to initial distribution of these nanoparticles within or adjacent to intracranial human xenograft tumors in nude mice, and a significant increase in animal survival [129].

Interestingly, the trafficking mechanism of antibodies bound to EGFR has been found to be different from that of EGF. Trastuzumab, also known as Herceptin®, is a monoclonal antibody that specifically binds to EGFR. In SKBr3 breast cancer cells, internalized Trastuzumab was highly colocalized with the recycling marker Tf, and was degraded quite slowly [130]; however, internalized EGF was sorted differently from Tf [131] and was more rapidly degraded [132].

VEGFR is overexpressed on the surface of a variety of tumor cells and on tumor neovasculature in situ, and is therefore considered a drug delivery target [133]. Docetaxel-loaded nanostructured lipid carriers conjugated with antibodies against VEGFR-2 exhibited superior cytotoxicity against cancer cell lines compared with non-targeted analogs. They showed better tolerance and antitumor efficacy in a mouse model bearing B16 melanoma compared with non-targeted analogs, which was attributed to the increased accumulation of drug in both tumor and tumor vasculature [133].

3.3. G-protein coupled receptor (GPCR) nanoparticle targeting

GPCRs represent the largest family of receptors, and serve as the targets of over 40% of the drugs approved by the FDA; however only a small number of GPCRs have been explored for targeting purposes [134, 135]. The ligands for GPCRs involve broad categories, including peptides, neurotransmitters, amino acids, hormones, lipids and chemokines [135]. GPCRs have two major classes of ligands: agonists that lead to receptor internalization upon binding and antagonists that do not [136]. Internalization is largely due to clathrin-mediated endocytotic pathways although there is considerable complexity and diversity to the pathways [137]. The polymorphisms and genetic mutants of GPCRs in humans may also increase the complexity of developing drug delivery strategies targeting GPCRs [138].

Theoretically, GPCRs could serve as gateways for nanoparticle binding and cellular uptake because of their high tissue specificity [136, 139]. Intriguing work targeting GPCRs was carried out on MCF-7 breast cancer cells expressing the human Y1-receptor, using quantum dots conjugated with agonist-type or antagonist-type peptide ligands [136]. Both agonist- and antagonist-modified quantum dots had excellent binding specificity, but exhibited a tremendous difference in cellular uptake: specifically, only agonist-modified quantum dots were internalized. Interestingly, these modified quantum dots bound to several receptor molecules simultaneously, and such multi-ligand binding characteristics can lead to

significantly elevated affinities by 5 orders of magnitude compared with free ligand. These important discoveries may pave the way for in-vivo application of drug carriers targeting GPCRs in the near future.

3.4. Folate receptor (FR) nanoparticle targeting

Overexpression of FR occurs in many human malignancies, especially aggressively growing cancers, and is associated with poor disease prognosis [140–142]. The FR has two glycosyl phosphatidylinositol (GPI)-anchored isoforms: α and β . FR- α is frequently over-expressed in epithelial cancers, whereas FR- β expression is found in myeloid leukemia and activated macrophages associated with chronic inflammatory diseases [143]. Based on the natural high affinity of folate for the folate receptor, drug carriers conjugated with folate may also bind tightly to the folate receptor and trigger cellular uptake via endocytosis [144]. As a GPI-AP, folate receptor internalization is thought to use clathrin-independent CLIC-GEEC internalization pathways [104, 145, 146].

Compared with antibodies, small molecules like folate are much easier to be manufactured at an industrial scale, which is an apparent merit of folate targeting for clinical application. For instance, an amphiphilic peptide conjugated to folate can be mixed with polymers to form nanoparticles that are capable of encapsulating DNA for gene delivery [147, 148]. The uptake of folate-conjugated nanoparticles by cell lines overexpressing folate receptors can be inhibited by competition with free folate, indicating the specificity of folate-targeting [147, 148].

Folate targeting has been extensively studied for its application in *in vivo* delivery of drug carriers [142, 144, 149]. Folate-targeted nanoparticle delivery of paclitaxel and the radio-therapeutic yttrium-90 (^{90}Y), showed significantly higher potency than the non-targeted analogs in *in vivo* efficacy studies using an ovarian peritoneal metastasis mouse model [149]. PEGylated liposomal doxorubicin infused with folate showed better activity relative to free doxorubicin in tumor models induced by human nasopharyngeal epidermoid carcinoma cell line, KB, and its vincristine-resistant derivative (KBV), both of which overexpress the folate receptor [144].

Lu *et al.* [150] used poly-lactic acid (PLA)-derived polymers to prepare folate-functionalized nanoparticles, which were labeled with ^{123}I to investigate their *in vivo* distribution. In BALB/c nude mice injected with folate receptor-positive HeLa cells, intravenously injected nanoparticles generated strong signals in the lung, liver, kidney, and in the xenograft tumors. The time-dependent signal intensity in the tumors suggested specific folate-targeting to tumors by drug-loaded nanoparticles; whereas the nanoparticles without folate showed high nonspecific accumulation in the liver [150]. In contrast, nano-sized siRNA complexes bearing folate did not significantly accumulate in tumors formed from KB cells, but exhibited a moderate silencing effect *in vivo* on the mitotic kinesin Eg5 gene [151].

Interestingly, the application of folate targeting is not limited to solid tumors, but also leukemia cells [143]. FR- β is expressed in approximately 70% of the cases of acute myelogenous leukemia (AML) blast cells [152]. FR- β -targeted liposomal doxorubicin showed higher in-vitro toxicity than its non-targeted analog in MV4–11 (human acute myelocytic leukemia) and K562 (human erythromyeloblastoid leukemia) cell lines. It also showed stronger inhibition in a colony formation assay with MV4–11, K562 cells and AML patient cells than its non-targeted analog [152]. This suggests the potential clinical application of folate targeting of liposomal nanoparticles in treating AML.

3.5. Integrin nanoparticle targeting

Integrins are heterodimeric transmembrane receptors that mediate the interactions between endothelial cells and the extracellular matrix [153]. They are involved in a large number of fundamental intracellular processes, such as cell-cell and cell-matrix adhesion, differentiation, stress responses and apoptosis [154]. This diverse family of receptors consists of at least 18 α and 8 β subunits that can dimerize in more than 24 different combinations to yield surface receptors capable of recognizing one or more extracellular matrix (ECM) components [155]. The resulting crosstalk with growth factors and cytokines regulates intracellular signaling that is essential in controlling cell survival, proliferation, and migration, whereas interaction of integrins with ECM provides the traction necessary for cell motility and invasion. Their internalization during cell migration and focal adhesion disassembly is thought to largely proceed by clathrin-mediated endocytosis [156, 157] although additional evidence suggests that different integrin subtypes may use other endocytotic mechanisms such as caveolar endocytosis under some conditions [158].

In solid tumors, integrins serve as regulators of the migration and invasion of tumor cells [159]. Expression of the integrins $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_5\beta_1$, $\alpha_6\beta_4$, $\alpha_4\beta_1$ and $\alpha_v\beta_6$ is correlated with disease progression in various tumor types [159]. In addition, 9 out of 24 integrin heterodimers have been implicated in blood vessel formation: $\alpha_1\beta_1$ [160–162], $\alpha_2\beta_1$ [160–162], $\alpha_4\beta_1$ [163], $\alpha_5\beta_1$ [164–168], [169], [170–175], [170, 171, 173], $\alpha_v\beta_1\alpha_v\beta_3\alpha_v\beta_5\alpha_v\beta_8$ [171, 176–178] and $\alpha_6\beta_4$ [179, 180]. Among these, $\alpha_v\beta_3$ receptors are overexpressed in endothelial cells undergoing angiogenesis, although they are not typically found on quiescent cells [181, 182]. This renders them attractive antitumor targets, since antagonists of this receptor that effectively compete with its natural ligand cause apoptosis in proliferating vessels [182].

The physical role of $\alpha_v\beta_3$ integrin is thought to involve matrix remodeling and degradation during endothelial cell migration. Matrix metalloproteinase-2 (MMP-2) is known to bind $\alpha_v\beta_3$ integrin in its active form, therefore allowing migrating endothelial cells to actively remodel the extracellular matrix [183]. Degradation of the native collagen by MMP-2 exposes otherwise unavailable $\alpha_v\beta_3$ integrin binding sites, facilitating endothelial cell invasion of the extracellular matrix. This model of endothelial cell migration is consistent with well-studied models of fibroblast migration wherein fibronectin receptors serve as “molecular feet” that bind their substrate at the leading edge of the cell and remain fixed as the cell moves forward.

A search for high affinity ligands for integrin receptors resulted in a number of peptidic [184, 185] or small molecule ligands [186]. Of particular interest to angiogenic therapies are the cyclic RGD peptides that have shown high affinity and specificity for α_v integrins. These ligands have been shown to selectively target tumor vasculature when injected into tumor bearing mice [187]. The specificity exhibited by peptides containing an RGD motif or small molecules resulting from combinatorial screen is remarkable given the relatively small molecular interface they present for binding to integrin receptors. Given that integrins are typically concentrated on the apical surface of migrating cells, a targeting approach that utilizes integrin-binding ligands is plausible. From a design perspective, such ligands need to be covalently linked to nanoparticles that serve as drug delivery vehicles. In addition to acting as more efficient delivery agents, it is also possible that such multivalent nanocontainers would be capable of interfering with the endocytotic cycle of $\alpha_v\beta_3$ integrin or the angiogenic process itself.

A synergy of integrin-mediated signaling with growth factor response was suggested over a decade ago. Ruoslahti *et al.* has found that engagement of the $\alpha_v\beta_3$ integrin in cell–matrix interactions also coordinates a strong response to growth factors, underscoring the

importance of this integrin for tissue regeneration, angiogenesis and tumor metastasis [188]. An involvement of $\alpha_v\beta_3$ integrins in insulin signaling was discovered when IRS-1, a cytoplasmic signal transduction mediator of the insulin and insulin-like growth factor 1 (IGF-1) receptors, was found to co-immunoprecipitate with $\alpha_v\beta_3$ integrin [188].

Later, a 100 kDa protein, identified as the insulin receptor (IR) beta chain by immunoprecipitation of complexes of $\alpha_v\beta_3$ integrin with anti-IR antibodies, followed by blotting with anti-phosphotyrosine (anti-pY) antibodies [189]. The integrin association typically requires growth factor stimulation of the receptors.

IGF-1 plays a key role in macrophage migration during the onset of tissue inflammation. IGF-1 induced recruitment of $\alpha_v\beta_3$ integrins, as well as translocation of the integrin adaptor protein phospho-paxillin to focal adhesion sites is also documented [190]. Experiments with pharmacological inhibitors of Akt, PKC and p38 MAP kinase revealed that IGF-1 facilitated migration requires IGF-1/IGF-1R-mediated PI3-kinase/PKC/p38-dependent integrin inside-out signaling, but does not require MMP activation. Similarly, $\alpha_v\beta_3$ integrin localization in the core of focal adhesions and involvement of $\alpha_v\beta_3$ integrin signaling pathways was found in an IGF-1 mediated migration of extravillous trophoblast (EVT) cells [191].

Doxorubicin-loaded human serum albumin nanoparticles conjugated with an antibody targeting $\alpha_v\beta_3$ -positive M21 melanoma cells exhibited enhanced cytotoxicity as compared to free doxorubicin [192]. However, there is a practical alternative other than using expensive antibodies: ligands containing an RGD tripeptide active site can be recognized by all five α_v integrins, two β_1 integrins (α_5 , α_8) and $\alpha_{IIb}\beta_3$ [193], and they are therefore useful tools for targeting integrins. When PEGylated-copolymer-coated iron oxide nanoparticles conjugated with a cyclic peptide containing this RGD sequence was administrated in mouse model for targeting $\alpha_v\beta_3$, successful tumor homing in-vivo was perceived in a subcutaneous U87MG glioblastoma xenograft model by magnetic resonance imaging (MRI) imaging [194]. Similar integrin-specific binding was achieved on human umbilical vein endothelial cells by paramagnetic liposomes conjugated with the cyclic RGD peptide [195]. siRNA encapsulated into RGD-labeled chitosan nanoparticles led to targeted silencing of multiple growth-promoting genes in nude mice bearing SKOV3ip1, HeyA8, and A2780 cells [196]. RGD-modified adenovirus expressing a therapeutic thymidine kinase suicide gene and a somatostatin receptor complexed to In-111 pentetateotide has demonstrated safety, potential efficacy, and possible gene transfer imaging capability in a Phase I clinical trial [197]. These studies support the possible clinical application of RGD-integrin targeting for imaging tumors and treatment of angiogenesis.

3.6. Transferrin receptor (TfR) nanoparticle targeting

As mentioned previously, Tfs are glycoproteins that bind free iron in the serum, and facilitate iron uptake into proliferating cells by binding to TfR on the cell surface. TfR is overexpressed in malignant cells due to the increased requirement for iron [198]. Upon binding to Tf, TfR is endocytosed into acidic compartments where the iron dissociates. This characteristic makes TfR a valuable target for compartment-specific drug delivery, particularly in cancer treatment.

Active targeting with PEGylated gold nanoparticles conjugated with Tf led to internalization into tumor induced Neuro2A cancer cells that have abundant TfR expression [116], exhibiting satisfactory TfR-targeting specificity *in vivo*. Comparison of the efficacy of the anti-cancer drug, hydroxycamptothecin, loaded into different drug carriers against sarcoma-180 tumors in mice showed that PEGylated non-ionic surfactant-based liposomes conjugated with Tf had significantly stronger inhibition of tumor growth compared with non-targeted PEGylated liposomes, non-coated liposomes and free hydroxycamptothecin.

Moreover, such efficacy was achieved without any side effect, such as decreased body weight [199]. A similar study compared the efficacy of doxorubicin loaded into stealth liposomes against Hep5 tumors in mice [200]. It also showed that PEGylated liposomes conjugated with Tf inhibited the tumor more potently than the non-targeted analogs. These studies all demonstrate increased drug delivery efficiency *in vivo* by Tf targeting. A strong advantage of Tf targeting, as opposed to use of signaling ligands, is due to its lack of complicating downstream signaling effects since it functions largely as a nutrient uptake mechanism.

Two TfR targeting liposomal systems have entered clinical trials [201]: MBP-426, a liposome conjugated with Tf for the delivery of oxaliplatin, has completed a Phase I clinical trial (2006, NCT00355888). It has also reached a Phase Ib/II clinical trial in second-line patients with gastric, gastroesophageal, or esophageal adenocarcinoma (2009, NCT00964080). SGT-53 is a liposome encapsulating plasmid DNA coding for wild type p53, with the conjugation of an anti-TfR single-chain variable fragment (TfRscFv) for targeting TfR. SGT-53 is currently in a Phase Ib clinical trial (2007, NCT00470613) in patients with advanced solid tumors.

A recent study showed that siRNA encapsulated in polymer nanoparticles conjugated with Tf caused a specific gene inhibition (reduction in mRNA and protein) by an RNAi mechanism of action when administered via systemic administration in Phase I clinical trial [202]. This study performed on patients with metastatic melanoma is the first example of a dose-dependent accumulation of targeted nanoparticles in human tumors [202], indicating the effectiveness of TfR targeting in a clinical application.

3.7. Low-density lipoprotein receptor (LDLR) nanoparticle targeting

Certain rapidly proliferating cancer cells have a high demand for cholesterol for cell membrane synthesis and therefore overexpress the LDL receptor [203, 204]. Thus the LDL receptor is regarded as a potential target that may facilitate the delivery and endocytosis of drug carriers into cancer cells.

LDL-mediated targeting of liposomes to leukemic lymphocytes *in vitro* was reported as early as 1985 [3]. Recently, siRNA delivery by LDL nanoparticles also showed enhanced gene silencing in HepG2 cells compared to their analog without LDL [205]. *In vivo* studies of LDL-receptor targeting also have given promising results: nanoparticles conjugated with LDL and near-infrared dyes showed specific tumor-targeting functions in mice, suggesting their value in enhanced optical cancer imaging [206]. Use of this receptor, like TfR, is theoretically less prone to complicating side effects since it too involves nutritional uptake.

4. Multivalent targeting

Multivalency is a term that describes the simultaneous interaction of multiple recognition elements on one molecular entity with multiple receptors on another entity. Multivalent ligands present multiple copies of the same recognition element (homomultimer) or a combination of different recognition elements (heteromultimer) that are connected via, or displayed upon, the central scaffold. The recognition element of a multivalent ligand can be a carbohydrate, peptide, protein, or small molecule – any moiety that binds to a receptor. The scaffold is the main determining factor of the structure of the multivalent ligand – it also dictates how easily the structural features can be varied. The most critical structural features are the shape, flexibility, size, valency and orientation of the individual recognition elements – they often dramatically influence activity and mechanism of action [207–210]. For example, in the processes that are activated by the receptor clustering that precedes endocytosis, the most potent multivalent ligands will be those that contain many closely

spaced recognition elements, whereas in the systems that rely on transcytosis, the scaffold size and selectivity of the interaction of each individual receptor-recognition element pair may also dictate the efficiency of its transport.

An important concept underlies the design of drug carriers based on multivalent ligands: many cell surface receptors are modular, with binding of the recognition element and signaling performed by structurally distinct and spatially segregated domains (Figure 4). Hence, interaction of the multivalent carrier with the extracellular region of the receptor may inexplicably activate the cell signaling cascade and alter the effect of the cargo. Because substrate binding and signaling activity are linked for many cell surface receptors, careful consideration of the receptor structure, receptor-receptor communication and crosstalk of the intracellular signals is required in order to orchestrate the overall response. For example, a multivalent display of the recognition element that is acting alone as an antagonist often leads to receptor clustering, resulting in use of the entire multivalent structure as an agonist [211]. Conversely, it has been proposed that multiple agonist recognition elements displayed in an orientation that holds receptors in an unproductive state for signaling conformations can result in a multivalent antagonist [212]. Likewise, ligand-induced intracellular sequestering of the signaling proteins can also result in an antagonist or agonist-like effects [213, 214].

Design of multivalent drug carriers can be accomplished through conjugation of multiple recognition elements onto an appropriate dendrimer, polymer, micelle or nanoparticle scaffold [215–217]. Overall, such constructs show enhanced binding affinity towards the target due to their distinct interaction mechanisms as compared to the monovalent ligand [209, 216, 218]. For example, ligands with multiple copies of a recognition element can bind to multiple binding sites on a single oligomeric receptor, such as an immunoglobulin or some lectins, by chelation or sub-site binding [219–221]. In both situations, the translational entropy cost is paid with the first receptor–ligand contact and subsequent binding interactions proceed without additional penalties in translational entropy [222]. For certain receptors that possess binding sub-sites in addition to their primary site of interaction, multivalent ligands may gain binding energy from contacting these secondary sites. In the situations where the multivalent ligand occupies more than one binding site—whether it is a subsite or a primary site within an oligomeric receptor—the ligand typically will bind with an increased affinity [223].

Another mechanism by which a multivalent ligand can act as an inhibitor is through steric stabilization; the steric bulk of the ligand may preclude the engagement of a bound receptor with an opposing viral particle or cell [224]. This aspect of multivalency is particularly attractive in the design of inhibitors of binding interactions between cellular and viral surfaces. Still another mode of interaction involves ligand-induced clustering of the multiple receptors on the cell surface, a process that is facilitated in the fluid lipid bilayer where two-dimensional diffusion of receptors is readily observed. Such clustering of the receptors often activates intracellular signaling and is extensively studied for the interaction of the integrin receptors with multivalent ligands with RGD-type peptide as recognition elements (*vide supra*) [225–227]. Lastly, statistical rebinding [228], where bulky multivalent ligands with low diffusion rate first dissociate but then bind again with the different recognition element to the same single-site receptor represents another interaction mechanism that can lead to an increase in binding affinity.

Homomultivalent ligands display multiple copies of the same recognition element and are often misclassified through the use of the more general term “multivalent”. The multiple recognition elements can be either directly conjugated to a drug delivery vehicle, or to a backbone that is, in turn, attached to a delivery vehicle. In both situations, the spacing of the

ligand, its orientation, the chemical nature of the linker backbone, and the length of the linker all have been shown to influence binding kinetics and binding cooperativity. For example, early studies by Kessler and coworkers, homodimers of somatostatin analogs conjugated to aliphatic linkers and spaced 16–18 angstrom apart showed 150 times increased affinity over the monomer, but as the linker length varied, the apparent affinity of the homodimers decreased [229]. Using multiple copies of α -melanocortin stimulating hormone tethered via spacers of different length and rigidity, Hruby, Gillies *et al.* showed that the structure of the spacer, its length, conformational flexibility and the orientation of the recognition element have an impact on the binding affinity of the ligand [230]. In addition, authors noticed that the enhanced cooperativity of the multivalent interaction (larger Hill coefficient) is largely dependent on the number of recognition elements and less dependent on the properties of the spacer.

The reports of quantitative, accurate and systematic studies of the binding equilibrium between homomultivalent nanoparticles and their target receptors are rare, probably due to the inherent difficulty in performing such assays where high binding constants must be measured under equilibrium conditions in the presence of multiple secondary interactions. Nevertheless, recent report on binding of dendrimer-based folate nanoparticles to surface-immobilized folate binding protein (FBP) provided important insights into the nature of such interactions [231]. The authors have shown that dissociation constants (K_D) between the homomultivalent nanoparticle and FBP, as measured by surface plasmon resonance (SPR) experiments, are dramatically enhanced (~2,500- to 170,000-fold over folic acid) and largely dependent upon the optimum number of folate ligands per particle. Examination of SPR data revealed a linear increase in the equilibrium association constant (K_a) as a function of the number of folates, indicating that the association to the surface does not exhibit any cooperativity. However, the decrease in equilibrium dissociation constant (K_D) was exponential as a function of the number of folates, demonstrating the cooperativity upon dissociation from the surface. Furthermore, cell-based assays showed enhanced uptake of the higher-affinity nanoparticles in KB cells. Interestingly, the rate of endocytosis was similar for all nanoparticles regardless of the number of ligands, suggesting that the observed enhancement of K_D , through multivalency, not an enhanced rate of endocytosis, is the key factor contributing to the improved rate of uptake of these drug delivery platforms. The effect of the linker was not reported in this work, however, the follow-up study with homomultivalent nanoparticles based on methotrexates conjugated to the same dendrimer suggests that the optimum length and conformational flexibility of the linker is important in functional assays such as inhibition of the catalytic activity of dihydrofolate reductase [232].

Multivalent ligands can also consist of dissimilar recognition elements. Such constructs are termed heteromultivalent and can recognize and simultaneously bind different types of cell surface receptors, potentially providing higher specificity toward a cellular phenotype that displays a particular receptor combination [233–236]. Numerous examples of multivalent targeting have been reported, such as heteromultimers of Tf-TfR plus RGD-integrin, and aptamer-nucleolin/tenascin-C plus RGD-integrin combinations. Both approaches demonstrated high specificity for *in vitro* delivery of nanoparticles [237, 238]. Furthermore, multivalent targeting also showed higher *in vivo* effectiveness as compared to monovalent ligands: nanoparticles using Tf-TfR + RGD-integrin increased the retinal delivery of nanoparticles that were administered intravenously [239].

One particularly cogent example of the increased efficacy of heteromultivalent targeting involves the use of cyclic RGD peptide and antagonist peptide anginex (Anx) conjugated to paramagnetic or fluorescent liposomes in targeting of $\alpha_v\beta_3$ integrins and galectin-1, respectively [240]. Galectin-1 has been identified as an important factor in the signaling of endothelial cell proliferation, migration, and apoptosis and it was proposed as a molecular

marker for target-specific imaging of angiogenesis. Using optical techniques and MRI it was shown that the heteromultivalent approach produced synergistic targeting effects with dramatically elevated uptake of nanoparticles into endothelial cells as compared to single-ligand, homomultivalent targeting [240].

Multivalency is, without a doubt, an important concept guiding the design of nanoparticles for drug delivery. Due to the paucity of the reported examples and complex role of multivalent interactions in cell-nanoparticle recognition, there are no clear guidelines for the precise placement of the interacting ligands in order to achieve the desired rate of internalization and localization and to minimize the off-target effects that arise from nonspecific binding. Given the important role of the structure of the nanoparticle itself, ultimately the success of the multivalent targeting strategy may come from the integration of the nanoparticle design with parallel synthesis, resulting in a library of nanoparticles that can be subjected to a screen against different cell lines for the desired property, (specificity, rate of internalization, *etc.*). Such an approach could facilitate rapid development of functional nanomaterials that utilize multivalent interactions for applications such as targeting specific cell types, differentiating cell lines, and detecting distinct cellular states.

5. Conclusion

Considerable advances have been made from the time when potential of receptor-mediated endocytotic targeting inferred 25 years ago was realized with the first successful attempts to enhance drug internalization through ligand conjugation. However the complexities inherent in the trafficking pathways themselves, the possibility of alteration of trafficking processes depending on the ligand concentration and other factors such as multivalency, and the growing body of knowledge regarding the diversity of ways in which the scaffolds themselves, nanoparticles, interact with different cells and tissues will keep drug delivery scientists busy for some time to come.

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Abbreviations

AML	acute myelogenous leukemia
AP	adaptor protein
APCs	antigen-presenting cells
Arf	ADP-ribosylation factor
CLIC	clathrin independent carriers
CtxB	cholera toxin B
ECM	extracellular matrix
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EPR	enhanced permeability and retention
ER	endoplasmic reticulum

ESCRT	endosomal sorting complex required for transport
FBP	folate binding protein
FcRn	neonatal Fc receptor
FR	folate receptor
GEEC	GPI-anchored protein-enriched early endosomal compartment
GPCR	G-protein coupled receptor
GPI-AP	glycosylphosphatidyl inositol anchored protein
HFE	familial hemochromatosis protein
IGF	insulin-like growth factor
IgG	immunoglobulin G
LDL	low-density lipoprotein
LDLR	low-density lipoprotein receptor
MMP	matrix metalloproteinase
PACS1	phosphofurin acidic cluster sorting protein
pIgR	polymeric immunoglobulin receptor
Rabs	ras-like proteins from brain
SNARE	receptor for soluble N-ethylmaleimide sensitive factor attachment proteins
SPR	surface plasmon resonance
StxB	Shiga toxin B
SV40	simian virus 40
Tf	transferrin
TfR	transferrin receptor
TGF	transforming growth factor
TGN	trans-Golgi network
TTP	TfR trafficking protein
VEGFR	vascular endothelial growth factor receptor

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Figure 1. Distinct pathways for cargo internalization by cells

The internalization pathways discussed in this article are illustrated in this figure: Phagocytosis involves surface receptors and engulfs large particles through envelopment by the plasma membrane. Macropinocytosis generates large macropinosomes containing extracellular fluid and soluble protein. Clathrin-dependent endocytosis involves the assembly of clathrin and adaptor proteins on a region of the plasma membrane in which particular receptors are clustered to form a nascent vesicle destined for internalization. Caveolin-dependent endocytosis involves the assembly of caveolin coats on regions of the plasma membrane rich in particular lipid rafts to form a nascent vesicle destined for internalization. CLIC is involved in the endocytosis of GPI-anchored proteins, and it is also a major contributor to fluid and bulk membrane uptake by cells. However, not all CLIC cargo have formally been shown to traffic to GEECs.

Figure 2. Bi-directional transcytosis of FcRn receptor in polarized epithelial cells

A) The pH-sensitive binding of FcRn to IgG occurs at pH < 6.5, but not at the physiological pH of 7.4. FcRn in the apical membrane of an epithelial cell can bind to IgG present in acidic luminal fluids for subsequent internalization and release of IgG at the basolateral membrane. B) Without a pH gradient across the polarized cells, e.g. alveolar epithelial cells or placental syncytiotrophoblast cells, FcRn is capable of transporting IgG bidirectionally. IgG can be endocytosed by fluid-phase uptake. Endocytosed IgG is then transported to an acidified endosomal compartment, in which the FcRn resides, and then binds to FcRn. The FcRn-IgG complex can then be transcytosed to the side contralateral to that which endocytosed the IgG.

Receptor Category	Receptor Subtype	Targeting molecule	Carrier/Vehicle	Cargo	Target Cells/Tissues	Reference
Receptor tyrosine kinase	EGFR	EGF	gelatin nanoparticle	cisplatin	A549 human lung adenocarcinoma tumor	[128]
	EGFRvIII	Antibody	iron oxide magnetic nanoparticles		glioblastoma multiforme (GBM) tumor	[129]
	VEGFR	Antibody	nanostructured lipid carriers	docetaxel	B16 mouse melanoma tumor	[133]
GPCR	Y1-receptor	Agonist/Antagonist	quantum dots		MCF-7 breast cancer cell line	[136]
Folate receptor		Folate	polymer nanoparticle	DNA	Calu-3 cell line, KB cell line	[147,148]
		Folate	polymer nanoparticle	⁹⁰ Y, paclitaxel	SKOV-3 cell line, SKOV-3 tumor	[149]
		Folate	PEGylated liposome	doxorubicin	KB cell line, SKOV-3, KB and KBV tumors	[144]
		Folate	polymer nanoparticle	¹²⁵ I	HeLa tumor	[150]
		Folate		siRNA complex	KB tumor	[151]
	FR-β	Folate	liposome	doxorubicin	MV4-11 and K562 human leukemia cell line, AML patient cells	[152]
Integrins	α _v β ₃	Antibody	human serum albumin nanoparticles	doxorubicin	M21 melanoma cell line	[192]
		RGD sequence	PEGylated-copolymer-coated iron oxide nanoparticles		U87MG glioblastoma tumor	[194]
			chitosan nanoparticles	siRNA	SKOV3ip1, HeyA8, and A2780 tumors	[196]
			Adenovirus	DNA	Recurrent gynecologic solid tumor in Phase I clinical trial patients	[197]
TfR		Transferrin (Tf)	PEGylated gold nanoparticles		Neuro2A neuroblastoma tumor	[116]
			PEGylated non-ionic surfactant-based liposome (niosome)	hydroxycamptothecin	sarcoma-180 tumor	[199]
			stealth liposome	doxorubicin	HepG2 human hepatocellular carcinoma tumor	[200]
			liposome	oxaliplatin	Adenocarcinoma in Phase Ib/IIa clinical trial patients	NCT00964080
			polymer nanoparticle	siRNA	metastatic melanoma solid tumor in Phase I clinical trial patients	[202]
		TfRscFv	liposome	DNA	Solid tumors in Phase Ib clinical trial patients	NCT00470613
LDLR		LDL	LDL nanoparticle	siRNA	HepG2 cell line	[205]
			LDL nanoparticle	near-infrared dyes	HepG2 human hepatocellular carcinoma tumor	[206]

Figure 3. Examples of cellular targeting strategies

In this table, the term “tumor” indicates tumors induced by injected cells in nude mice.

Figure 4. Multivalent Targeting of Integrin Receptors Activates Signaling

Phase 1, the cargo bearing ligands approaches the plasma membrane bearing receptors; Phase 2, the ligands bind the recognition domain of the receptors and trigger polymerization of receptors; Phase 3, the spatially segregated intracellular domains of the receptors initiate downstream signaling; Phase 4, the cargo falls into the invagination formed by the plasma membrane; Phase 5, the cargo is internalized for subsequent intracellular trafficking. Binding of nanoparticle to integrins can activate signaling pathways and subsequently affect cell proliferation, differentiation or migration. Integrins synergize with other cell surface receptors, such as receptor protein tyrosine kinases, to activate signaling via ERK1/2 cascade. ILK – integrin-linked kinase, FAK – focal adhesion kinase, RTK – receptor tyrosine kinase.